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Molecular typing and colonization patterns of *Aspergillus fumigatus* in patients with cystic fibrosis

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Abstract

Aspergillus fumigatus is a chronic colonizer of the respiratory tract of patients with cystic fibrosis (CF). A total of 204 *A. fumigatus* isolates from 36 CF patients from three different medical centers, collected over a period of four months till 9.5 years, were genotyped using the short tandem repeat panel for *A. fumigatus* (STR*Af* assay). Four different colonization patterns were observed. Colonization patterns with only unique genotypes were found in 36% of the patients. In contrast 17% of the patients were chronically colonized with a single genotype. The remaining patients showed a predominant genotype or genotypes that succeed each other. In this collection no relation was found between colonization patterns and allergic bronchopulmonary aspergillosis.

Keywords: *Aspergillus*; Cystic fibrosis; Colonization; Molecular typing

1. Introduction

Cystic fibrosis (CF) or mucoviscidosis is an autosomal recessive disorder with an incidence of approximately one in 2500 live births in the Caucasian population. Mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) result in dysfunction of the exocrine glands. As a consequence of this deficient transport mechanism, copious amounts of viscous respiratory mucus are secreted, which is difficult to clear and provides a breeding ground for microorganisms [1]. Early in life, CF patients become infected with a limited spectrum of bacteria, mostly *Staphylococcus aureus* and *Haemophilus influenzae*. As the disease progresses, *Pseudomonas aeruginosa* becomes the most common pathogen [2]. *Aspergillus fumigatus* is by far the most isolated filamentous fungus from respiratory secretions of CF patients and up to 57% are chronically colonized with this fungus [3]. A commonly recognized complication of *Aspergillus* infection in CF patients is allergic bronchopulmonary aspergillosis (ABPA), with a prevalence of approximately 1 to 15% [3–5]. Although persistent inflammation and the resulting pulmonary injury can finally progress to fibrosis [6], knowledge about the epidemiology of *A. fumigatus* in CF patients is scarce [7,8].

A better understanding of the airway colonization by *A. fumigatus* in CF patients may be achieved by accurate molecular typing of sequential isolates from sputum samples. For *A. fumigatus*, several typing techniques have been described previously, including microsatellite based fingerprinting assays. The STR*Af* assay, based on nine microsatellite or short tandem repeat (STR) markers, is a robust [9], reproducible [10] typing technique with a high discriminatory power [11]. These

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characteristics differentiate the STR based assays from many other previously employed typing techniques, such as random amplified polymorphic DNA (RAPD) [12] and restriction fragment length polymorphism (RFLP) [13], which suffer from poor interlaboratory reproducibility and subjective interpretation of the fingerprinting data [14].

Up to now, four studies comprising a total of 20 CF patients have described the colonization pattern of *A. fumigatus* [15–18]. Verweij et al. [18] performed molecular typing of sequential isolates from two patients with RAPD. With the *Afut*1-RFLP method, a predominant genotype in sequential sputum samples of one patient was detected. In another patient nine different genotypes were seen in 12 sputum samples. Neuveglise et al. [16] collected 412 *A. fumigatus* isolates from 6 patients over a 2.5-year-period. These isolates yielded 54 unique genotypes with the *Afut*1-RFLP method. In all samples of the six patients, several *A. fumigatus* genotypes were found, whereas two patients harbored a predominant genotype. The two other studies [15,17] used solely the low discriminatory molecular typing assay RAPD, which suggested the majority of isolates to be of the same genotype [15].

In this study we report the colonization patterns of 196 *A. fumigatus* genotypes in 36 different CF patients from 3 European medical centers. The isolates were collected from sequential sputum samples and analyzed using the highly discriminatory STR*Af* assay.

2. Materials and methods

2.1. Isolates

A total of 204 isolates from 36 CF patients were included in this study. Sputum samples were collected from these patients from 1996 through January 2008 during routine clinical visits or during an admission to hospital because of exacerbation of their pulmonary disease. The time period between the first and the last *A. fumigatus* isolate per patient varied from four months to nine and a half years. The numbers of isolates per patient varied from two till 27. The patients originated from three different centers; 100 isolates from 14 patients were obtained from the Cystic Fibrosis Center Dekkerswald (Nijmegen, The Netherlands); 58 isolates from 15 patients were collected from the University of Aachen (Aachen, Germany); another 46 isolates from 7 patients were from the University Medical Center Würzburg (Würzburg, Germany).

2.2. Collection of A. fumigatus isolates

Sputum samples obtained from patients were routinely cultured on Sabouraud agar for 7 days at 30 and 35 °C. *A. fumigatus* isolates were identified at the time of collection by their macroscopic and microscopic characteristics and their ability to grow at 48 °C. The obtained isolates were stored as spore suspensions in regular microbial freezing broth containing 12.5% (vol/vol) glycerol at −80 °C until testing. The isolates were revived by scraping off part of the frozen broth, plated on Sabouraud or potato agar, and cultivated at 30 °C.

2.3. STRAf assay

Genomic fungal DNA was extracted and purified from all isolates with the MagNA Lyzer and MagNA Pure LC Instruments (Roche Diagnostics, Almere, The Netherlands) and PCR primers and conditions were exactly as described before [11]. The obtained PCR fragments were diluted 30 fold with distilled water. One µl of diluted PCR products was combined with 0.25 µl of ET-ROX 400 marker (GE Healthcare, Diegem, Belgium) and denaturated for 1 min at 95 °C followed by rapid cooling to 4 °C in a total volume of 10 µl. The denaturated fragments were analyzed on a MegaBACE 500 automated DNA analysis platform (GE Healthcare), according to the manufacturer's instructions.

2.4. Data analysis

Electropherograms were analyzed using Fragment Profiler 1.2 software (GE Healthcare). Identical isolates were those that possessed alleles with the same number of repeat units in all nine loci. Isolates with genotypes that differed in a single locus were considered to be genetically related. The term unique was used for genotypes that were only found once in a patient.

3. Results

A. fumigatus isolates from 36 CF patients were analyzed using the STR*Af* assay. For 196 of the 204 isolates, a single genotype was obtained. Eight samples displayed multiple peaks for all nine loci, indicating to be a mixture of two or more different *A. fumigatus* isolates. An overview of the remaining 196 genotypes is shown in Fig. 1 and described below.

Six CF-patients (#1, #6, #8, #9, #17 and #29) harbored only identical or related *A. fumigatus* isolates. From five of these patients, two to four isolates were cultured with a maximum time interval of 1.5 years. Moreover, patient #29 even showed 7 related isolates over a 3.5 years period. From 7 patients (#3, #7, #16, #18, #26, #34 and #35) most of the isolates were identical, since all patients had one or two unique isolates besides the predominant genotype. Two of the three isolates from patient #35 were identical, the time interval between these two isolates was 9.5 years.

In three patients (#25, #28 and #32), identical isolates were found successively in a period of about one year. The isolates cultured before or after this period demonstrated unique genotypes. Patients #27 and #30 also showed identical and unique isolates. However in these patients during a period in which identical genotypes were found, also unique genotypes were present. For example, genotype P from patient #27 was found in month 11, two years later three other genotypes were found in the sputum samples of this patient, but after three years genotype P appeared again.

In the following group of patients, several genotypes were found more than once. For instance, in patient #20 genotype M was found twice, followed by genotype N that was also found twice. In patient #36 a genotype '**ε**' was found four times in a period of 9.5 years, whereas second genotype '**λ**' was observed

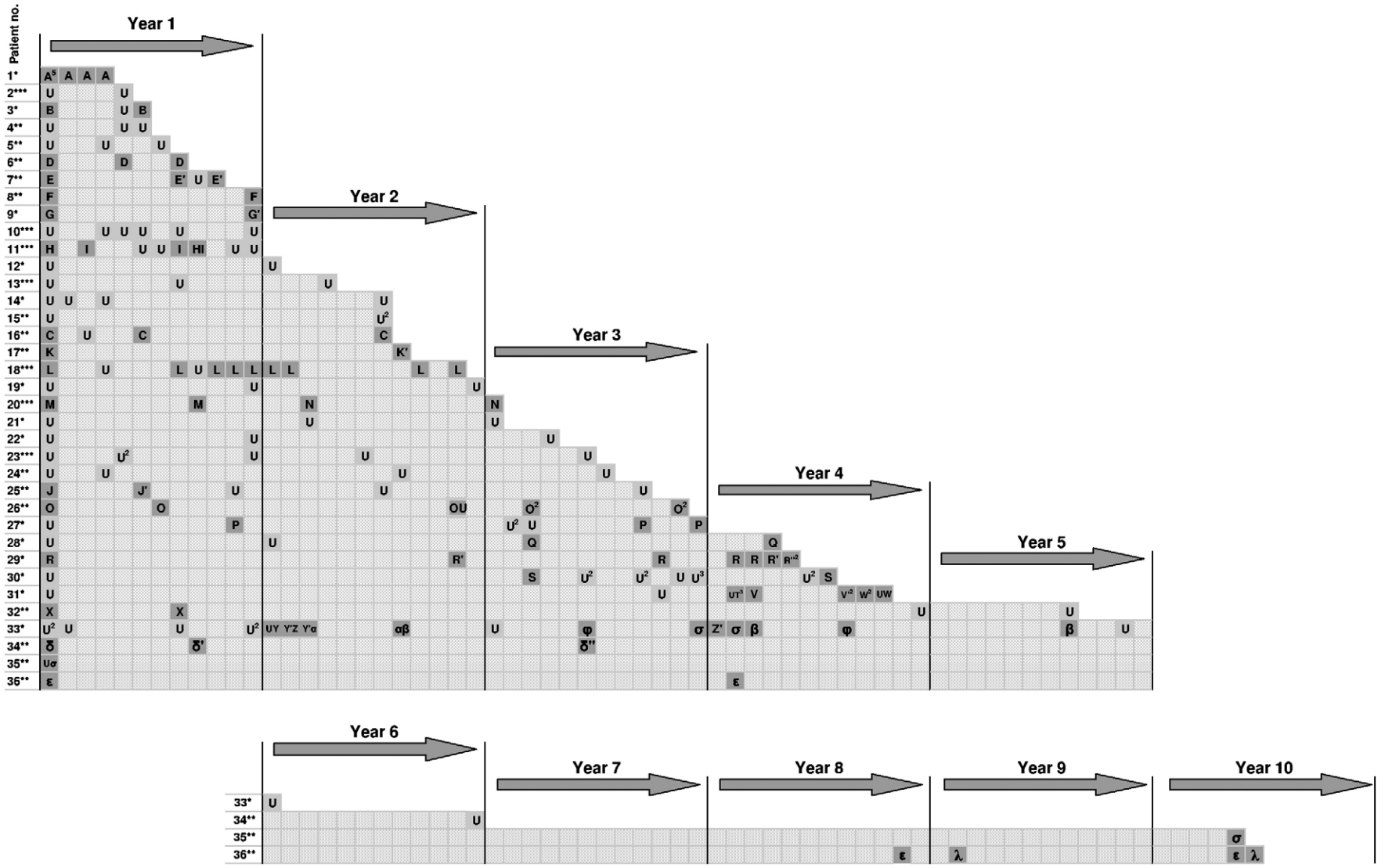

Fig. 1. Graphical representation of the genotyping results of *A. fumigatus* isolates from 36 CF patients. Each patient is represented horizontally (numbers 1–36). The "*" symbol indicates patients originating from the Cystic Fibrosis Center Dekkerswald, patient numbers indicated with "**" are from the University of Aachen and with "***" from the University Medical Center Würzburg. The arrows indicate one year whereas each square indicates one month. Unique isolates were depicted with the symbol U, these genotypes were only found once within a patient. Isolates from a patient possessing alleles with the same number of repeat units in all nine loci are identified by a unique symbol. The ′ symbol after a letter indicates a genotype that differed only in a single locus. The ^{2,3} and ^{5} after a symbol showed the number of isolates found within a month, for example U^{2} means that two unique isolates were found within one month's period.

after 8 years and onwards. In patient #31, three genotypes (T, V and W) were observed in successive time intervals varying from one to five months. They alternated with unique genotypes. Patients #11 and #33 demonstrated the most complex colonization patterns. The 9 isolates from patient #11 showed six different genotypes with two of these genotypes found more than once. The 27 isolates from patient #33 yielded 16 different genotypes, 6 of them were found more than once. The remaining 12 patients only showed unique genotypes.

4. Discussion

The *A. fumigatus* colonization patterns in 36 CF patients were investigated by typing 204 isolates with the STR*Af* assay, the largest study so far. From 196 *A. fumigatus* a genotype was obtained, the remaining isolates were mixed genotypes. In general, four different colonization patterns can be discriminated. First, continuous colonization patterns were found. The patients yielded identical or related genotypes for all isolates over a prolonged period of time, indicating that they were not able to clear the *A. fumigatus* isolate. In this collection 17% of the patients showed colonization with only one genotype. The second colonization pattern consisted of a predominant genotype, meaning that most of the isolates were identical. This group of patients were also not able to clear the *A. fumigatus* isolate and were occasionally co-colonized with a second genotype. This was the case in 19% of the patients. The third colonization pattern, found in 28% of the patients, contained genotypes which succeeded each other. These patients were eventually able to clear the *A. fumigatus* isolate, but were easily recolonized. The remaining 36% of the patients yielded colonization with only unique *A. fumigatus* isolates, indicating that these patients were continuously able to clear the fungus from the respiratory tract.

In some other studies the relation between colonization patterns of CF patients and clinical symptoms was investigated [16,18]. In our study population, we did not find an obvious correlation between colonization pattern and the development of ABPA, because only three CF patients in this study were diagnosed with ABPA. In a large multicenter study, 25% of patients aged 6 years or older harbored *Aspergillus* [19], yet the prevalence of ABPA in CF patients in the USA is only 2% [20]. Colonization seems to be related with prophylactic antibiotic use as a risk factor but there was no relation with lungfunction [21]. Patients using aerolized tobramycin had a higher frequency of acquisition of *Aspergillus* than did patients without tobramycin, but no difference existed in ABPA and fungal pneumonia [22]. To get more insight in relations between colonization patterns and clinical symptoms, the study should be expanded with a larger number of CF patients with proven ABPA.

In each center an identical genotype was found in two different patients and two different patients from two different centers also showed identical genotypes (data not shown). We were unable to find a common source of the *Aspergillus*. The significance of this finding remains unclear, since the highly discriminatory STR*Af* technique can separate a minimum of 99% of unrelated isolates. [11].

Molecular fingerprinting of *A. fumigatus* isolates from the environment and from clinical samples showed that a high extent of genetic variability can be found [23]. Therefore typing techniques with high discriminatory power are necessary to differentiate isolates from each other. Both the STR*Af* assay and the *Afut*1-RFLP method have shown to fulfill this criterion [11,23]. Individuals may constantly be exposed to a large variety of different genotypes; this is in concordance with the 36% of the patients where only unique genotypes were found. In the remaining patients identical genotypes were found over longer periods, which is even more surprising since these patients were also constantly exposed to different genotypes. In patients #35 and #36 identical genotypes were found for more than 9 years. It is likely that inside the CF lung a different environment (local immunosuppression, different pH and growth nutrition) is present compared to healthy lungs. This may cause a different growth and virulence of *A. fumigatus*. Beauvais et al. [24] and Mowat et al. [25] recently demonstrated that *Aspergillus* has the ability to produce a matrix that surrounds hyphae which protects from the action of antifungals. Future studies may show if the mucus and the self produced matrix cause a barrier against the human defense and antifungals and therefore lead to a longer persistence. However, despite the presence of the viscous mucus, 36% of the patients in our collection with a unique colonization pattern were able to clear the fungus from the respiratory tract.

The colonization patterns of *A. fumigatus* in CF patients was comparable with other patient categories. In previous studies successive *A. fumigatus* isolates were typed from non CF-patients. Several patients with invasive aspergillosis [26,27] and aspergilloma [28] were colonized with multiple genotypes, whereas other patients harbored unique genotypes. However, there is a difference between our study and the latter, since almost all of the isolates were collected within one month. Symoens et al. [29] collected *A. fumigatus* isolates from eight lung transplant recipients. From three of these patients several isolates were collected over a period of 1 to 3 years also showing a continuous, predominant and unique type of colonization. To compare the colonization patterns in CF patients with those with other underlying diseases, similar studies should be performed with more non CF patients colonized with *A. fumigatus*. Such a group could be the patients with other chronic respiratory diseases, like bronchial asthma and chronic obstructive pulmonary diseases, since these patients also are prone to develop ABPA [30].

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